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RESEARCH ARTICLE / *ARTIGO*

Isoenzymatic characterization of *Colletotrichum kahawae* isolates with different levels of aggressiveness

Andreia Loureiro[1,2], Leonor Guerra-Guimarães[1], Fernando C. Lidon[2], Benoit Bertrand[3], Maria C. Silva[1] & Vítor Várzea[1]

[1]Instituto de Investigação Científica Tropical, Centro de Investigação das Ferrugens do Cafeeiro (CIFC), Quinta do Marquês, 2784-505, Oeiras, Portugal; [2]Grupo de Disciplinas de Ecologia da Hidrosfera, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825-114, Caparica, Portugal; [3]Centre International de Recherches Agronomiques pour le Développement (CIRAD), UMR RPB, IRD-CIRAD-UM2, BP 64501-34394, Montpellier, France

Author for correspondence: Andreia Loureiro, e-mail: andreiasofialoureiro@gmail.com

ABSTRACT

The hemibiotrophic fungus *Colletotrichum kahawae* is the causal agent of Coffee Berry Disease - CBD, an economically devastating disease restricted to Arabica coffee production in Africa. Understanding pathogen variability is crucial for the implementation of disease control measures. In this study, six isoenzymatic systems (esterase, acid and alkaline phosphatase, malate dehydrogenase, peroxidase and superoxide dismutase) were used to assess the genetic variation among 12 *C. kahawae* isolates (from different geographic origins and with different levels of aggressiveness towards coffee) and one *Colletotrichum gloeosporioides* isolate (not pathogenic to green berries). Cluster analysis of the banding profiles obtained for the six enzymes enabled the differentiation of the two species and revealed the existence of intraspecific variability among *C. kahawae* isolates. Alkaline phosphatase was the most discriminative enzyme, allowing also the discrimination between the most and least aggressive isolates of *C. kahawae*.
Key words: Coffee berry disease, isoelectric focusing, isoenzymes, variability.

INTRODUCTION

Coffee berry disease (CBD), caused by the fungus *Colletotrichum kahawae* J. M. Waller & P. D. Bridge, is currently restricted to Arabica coffee (*Coffea arabica* L.) in Africa. CBD is still a quarantine disease with the foreseen threat of one day becoming a problem in Latin America and Asia, especially in high-altitude coffee producing areas where climatic conditions appear to be favourable for fungus development. During prolonged wet and cold weather, this disease can quickly destroy 50-80% of the developing green berries (Van der Vossen & Walyaro, 2009). *C. kahawae* is closely related to *C. gloeosporioides*, which also occurs (along with *C. acutatum*) as a saprophyte in the coffee mycobiota (Waller & Masaba, 2006). Considering that CBD is a major threat to Arabica coffee, the accurate and timely identification of the pathogen becomes extremely important to the coffee industry and to the maintenance of plant health (Bridge et al., 2008).

Like other *Colletotrichum* species, *C. kahawae* differentiation and variability has been studied considering morphological, cultural and pathogenic characteristics, vegetative compatibility groups (VCG) and also molecular and isoenzymatic studies (Omondi et al., 2000; Várzea et al., 2002; Derso & Waller, 2003; Silva et al., 2006; Bridge et al., 2008). Pathogenic tests using isolates from several African countries and various coffee genotypes revealed aggressiveness variation among *C. kahawae* isolates (Manga et al., 1997; Omondi et al., 2000). Small differences between East and West Africa isolates were detected when using VCG (Várzea et al., 2002; Bridge et al., 2008).

Low variability was observed when several molecular techniques, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP), inter-simple sequence repeat (ISSR), variable number of tandem repeats (VNTR) and amplified fragment length polymorphisms (AFLP) were used to study *C. kahawae* isolates (Derso & Waller, 2003; Bridge et al., 2008; Manuel et al., 2010). Recently, Silva et al. (2010), combining information from a multi-locus analysis (β-tubulin2, ITS and MAT1-2-1 genes), were able to discriminate *C. kahawae* isolates in three divergent but clonal geographical groups: Angola, Cameroon and East Africa, confirming the low genetic variability within *C. kahawae*.

Regarding *C. kahawae*, and despite the low variability found at the molecular level, preliminary studies based on α-esterase and acid phosphatase profiles obtained by isoelectric focusing (IEF) electrophoresis revealed the existence of some variability among isolates (Várzea, 1995; Loureiro et al., 2004). Isoenzymatic analyses were successfully used to study intraspecific variations and also

to establish interspecific differences among *Colletotrichum* species (Bonde et al., 1991, Rego et al., 1994; Lima & Menezes, 2002; Lima Filho et al., 2003; Horvath & Vargas, 2004).

By increasing the number of enzymes tested and using a different set of isolates from different geographical origins, the present study aimed to enhance the usefulness of isoenzyme analysis to determine the variation between *C. kahawae* isolates with different levels of aggressiveness. Six isoenzymatic systems were studied by IEF and each isolate aggressiveness was tested.

MATERIAL AND METHODS

Fungal isolates

Single spore isolates of *C. kahawae* obtained from coffee berries came from Angola (Ang6), Ethiopia (Eti17), Malawi (Mal2), Rwanda (Rua1), Tanzania (Tan1), Kenya (Que2, Que48, Que70, Que71, Que72), Cameroon (Cam1), and Zimbabwe (Zim1), and one isolate of *C. gloeosporioides* obtained from coffee berries came from China (Chi1). The isolates were maintained in malt extract agar (Oxoid) 3.4%, at room temperature.

Aggressiveness test

Detached expanding green berries of the variety Caturra were inoculated with *Colletotrichum* isolates grown at 25ºC, according to the technique described by Van der Vossen et al. (1976), with slight modifications. The green berries were placed on trays, lying down on a nylon sponge and inoculated with a 5 µL-drop of conidia suspension ($2x10^6$/mL), obtained from 10-day-old cultures. Covered trays were placed in a Phytotron 750 E (Aralab, Portugal) at 22ºC, incubated for the first 24h in the dark and then kept with a photoperiod of 12 h. The number of days between inoculation, the appearance of the first symptoms and the index of disease intensity (IDI) 10 days after inoculation were recorded. The IDI was calculated based on a 0 - 8 disease severity scale (Table 1), using the formula IDI = ∑ (number of green berries in each class x numeric value of each class) / (total number of green berries x eight). Thirty green berries were tested for each isolate. *C. gloeosporioides* was used as a negative control. The significance of aggressiveness was determined using Tukey's multiple range test ($P \leq 0.05$).

TABLE 1 – Scale of disease classification used to calculate the index of disease intensity

Class	Description
0	Green berries without symptoms
1	Black points in the inoculation spot (1-2 mm)
2	Black lesions with approximately 3 mm diameter
3	Black lesions with approximately 5 mm diameter
4	Black lesions with approximately 7 mm diameter
5	Black lesions with approximately 10 mm diameter
6	Black lesions with approximately 12 mm diameter
7	Black lesions with approximately 15 mm diameter
8	Whole berries covered with black lesions

Protein extraction

Enzymatic extracts were obtained from the 13 isolates, grown in 50 mL of liquid medium (malt extract 30% and peptone 5%) for 10 days at 25ºC, without agitation. Mycelium was collected on filter paper by vacuum filtration and washed several times with dH_2O, being thereafter homogenized with acetate buffer (0.05M, pH 4.5). The homogenate was centrifuged at 20000 g for 1 h at 4ºC. The supernatant was collected, dialyzed against water at 4ºC overnight, and concentrated in polyethylene glycol (6000). The extract was stored at -80ºC until electrophoretic analysis. The protein content of each sample was determined according to the Bio-Rad (USA) protein assay kit.

Electrophoresis and enzyme visualization

Six enzymatic activities were studied by IEF electrophoresis, in a vertical slab 1.5 mm thick with 5% (w/v) polyacrylamide gel and 2% ampholytes (Robertson et al., 1987): esterase (EST, EC3.1.1.2), alkaline phosphatase (ALP, EC3.1.3.1), acid phosphatase (ACP, EC3.1.3.2), malate dehydrogenase (MDH, EC1.1.1.37), peroxidase (POD, EC1.11.17) and superoxide dismutase (SOD, EC1.15.1.1). IEF was performed on a Mighty Small SE 250 apparatus (Hoefer, England) with electrode solutions containing 25 mM NaOH (catode) and 20 mM acetic acid (anode) for all the enzymes. Electrophoresis was performed at a constant voltage of 200 V for 50 min, followed by another 50 min at a constant voltage of 400 V.

After migration, EST, ALP, ACP, MDH, POD and SOD were stained according to Nave & Sauhey (1986), Guedes (1988), Scandalios (1969), Shawn & Prasad (1970), Smith & Hammerschmidt (1988) and Vallejos (1983), respectively.

Enzyme analysis

Data matrices from enzyme patterns were formed by identifying the presence (1) or absence (0) of a particular band. A genetic similarity matrix based on Jaccard's coefficient was calculated considering $S_J=a/(a+b+c)$, where a are bands present in both isolates being compared, b are bands present only in the first isolate and c are bands present only in the second isolate. A phenogram based on the estimated similarity coefficients was constructed by unweighed pair group method analysis (UPGMA), using the computer software package NTSYS-pc version 2.02 (Exeter Software, USA). The original similarity matrix was compared with the cophenetic value matrix generated from the systems of clusters. From the comparison of these two matrices, the cophenetic coefficient (r) was estimated and used as fitting measurement.

RESULTS

Aggressiveness test

All the *C. kahawae* isolates were virulent to the susceptible cultivar Caturra, causing dark sunken lesions, and the isolate Chi1 from *C. gloeosporioides* did not cause disease symptoms, as expected. The first disease symptoms occurred three days after inoculation on the green berries inoculated with the isolate Cam1 (Table 2). This isolate showed the highest IDI values (Table 2) and differed from the isolate Mal2 that only showed symptoms nine days after inoculation and displayed the lowest IDI (Table 2). The remained *C. kahawae* isolates formed a homogeneous group with IDI that did not differ from isolates Cam1 and Mal2.

Isoenzymatic analysis

The IEF electrophoresis of the six isoenzymatic systems were consistently visualized and clear patterns were detected for all the *Colletotrichum* isolates, and a total of 69 bands were assessed. For each enzyme tested the variation in the number of bands obtained per isolate and the respective range of isoelectric points were recorded (Table 3).

The EST isoenzymatic patterns showed a high number of bands per isolate, with high degree of polymorphisms, except for isolate Cam1 of *C. kahawae* for which only five bands were detected (Table 3 and Figure 1a). Based on the cluster analysis, isolates Cam1 of *C. kahawae* and Chi1 of *C. gloeosporioides* were clearly distinguished from the other isolates, and the values of the similarity coefficient were 23% and 38%, respectively (Figure 2a).

For ACP, the number of bands varied from four to six for the *C. kahawae* isolates while only three bands were found for the *C. gloeosporioides* isolate (Table 3 and Figure 1b). Only isolates Cam1 and Que72 of *C. kahawae* and isolate Chi1 of *C. gloeosporioides* exhibited POD activity (Table 3 and Figure 1c). Two groups were identified when the SOD isoenzymatic pattern was analyzed: one characterized by the presence of two isoforms (pI 4.6 and 5.9) and another with only one isoform (pI 5.9) (Figure 1d). ACP, SOD and POD profiles were not useful to separate *C. kahawae* from *C. gloeosporioides*, and displayed low polymorphism among *C. kahawae* isolates (data not shown). However, despite the polymorphism found in the MDH system, expressed by a variation from four to 12 bands per isolate (Table 3, Figure 1e), this enzyme was also not helpful to distinguish *C. kahawae* from *C. gloesporioides* (data not shown). In the ALP isoenzymatic patterns the number of identified bands varied between two and six, with a predominance of four on 70% of the isolates (Figure 1f). There was one common band to all isolates. For this enzyme, the cluster analysis showed (Figure 2b) a clear separation of *C. kahawae* and *C. gloeosporioides* (similarity coefficient of 15%). Among *C. kahawae* isolates, polymorphism was found with Cam1 and Mal2 showing a low similarity coefficient (38% and 52% respectively), compared to the remaining isolates (Figure 2b).

Cluster analysis of the 13 *Colletotrichum* isolates based on the banding patterns obtained for the six enzymes clearly separated the two species (similarity coefficient of 34%) (Figure 2c). Within cluster I, isolate Cam1 was clearly distinguished from the others (similarity coefficient of 37%). The remaining isolates of cluster I could be separated in two subgroups with a 50% similarity. Group Ia integrates several isolates from Kenya (Que2, Que48, Que71, Que72), Angola (Ang6), Zimbabwe (Zim1), Malawi (Mal2) and Rwanda (Rua1), and group Ib joins two isolates, one from Tanzania (Tan1) and another from Kenya (Que70). The high correlation coefficient (r=0.93) showed that the dendrogram structure is a strong indicative of phenetic relationship among all the isolates.

TABLE 2 - Appearance of the first symptoms on green berries inoculated with *Colletotrichum* isolates, and index of disease intensity (IDI) ten days after inoculation

Isolate	Appearance of the first symptoms (days after inoculation)	IDI
		25ºC (x±SD)
Ang6	4	0.23±0.09 ab
Cam1	3	0.44±0.15 b
Eti17	5	0.33±0.07 ab
Mal2	9	0.005±0.003 a
Rua1	4	0.21±0.08 ab
Zim1	4	0.22±0.09 ab
Que2	5	0.17±0.01 ab
Que48	5	0.25±0.05 ab
Que71	5	0.19±0.01 ab
Que72	5	0.27±0.09 ab
Chi1	-	-

[1]mean ± standard deviation. [2]According to Tukey's test (P≤0.05), in each column, different letters indicate significant differences.

TABLE 3 - Variation in the number of bands obtained per isolate and respective range of isoelectric points, for each enzyme tested

Enzyme	Number of bands (range)	Isoelectric point (range)
Esterase	5 to 15	3.4 to 7.9
Acid phosphatase	3 to 6	3.5 to 5.4
Alkaline phosphatase	2 to 6	4.2 to 5.4 and 7
Malate dehydrogenase	4 to12	4.7 to 8.6
Peroxidase	1 to 5	3.8 to 9.5
Superoxide dismutase	1 to 2	4.6 to 5.9

DISCUSSION

The most distinctive characteristic of *C. kahawae* that enables it to occupy a unique ecological niche, and which separates it on a functional basis from all the other

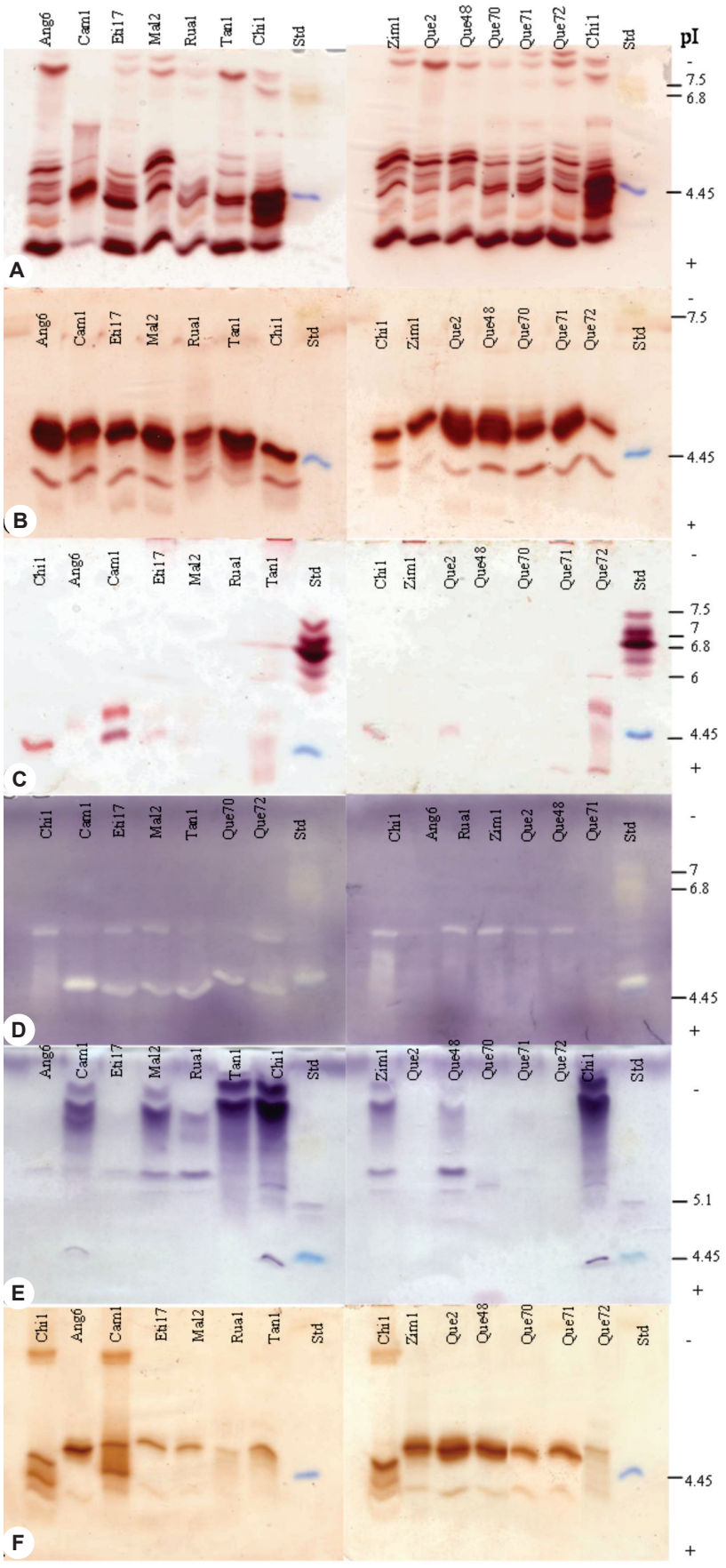

FIGURE 1 - A. Zymograms of esterase, **B.** acid phosphatase, **C.** peroxidases, **D.** superoxide dismutase, **E.** malate dehydrogenase and **F.** alkaline phosphatase isoenzymes of twelve *Colletotrichum kahawae* isolates: Ang6 (Angola), Cam1 (Cameroon), Eti17 (Ethiopia), Mal2 (Malawi), Rua1 (Rwanda), Tan1 (Tanzania), Zim1 (Zimbabwe), Que2, Que48, Que70, Que71, Que72 (Kenya) and one *C. gloeosporioides* isolate - Chi1 (China). Std – Isoelectric focusing standards broad range pI 4.45-9.6 (Bio-Rad). **A-B** and **F**. 10 µg of protein were loaded per lane. **C-D** and **E.** 20 µg of protein were loaded per lane. (-) Catode, (+) Anode.

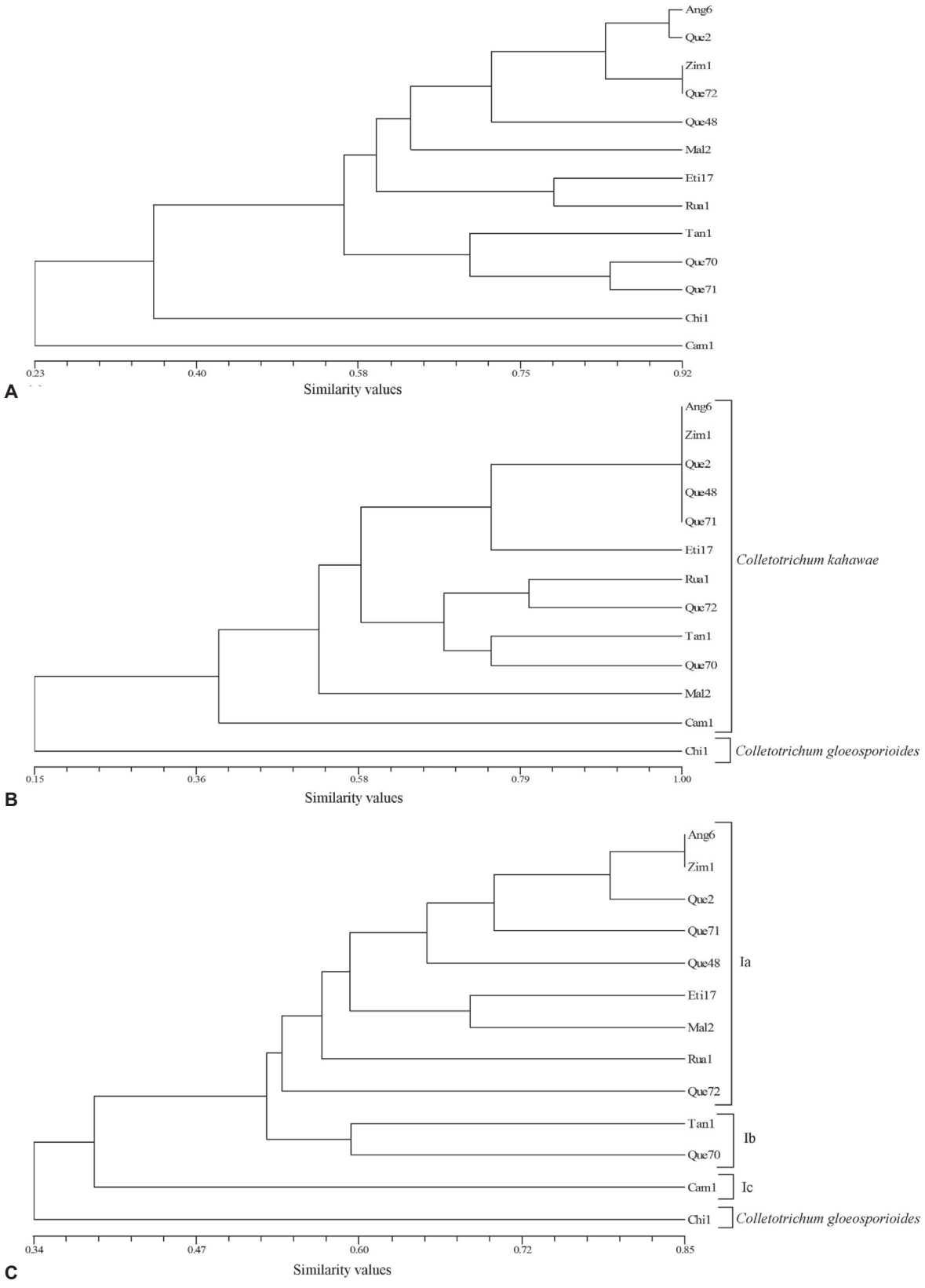

FIGURE 2 - Dendrogram resulting from cluster analysis of 12 *Colletotrichum kahawae* isolates and one *C. gloeosporioides* isolate, using Jaccard's similarity coefficient based on: **A.** esterase zymogram (cophenetic correlation coefficient r = 0.92819); **B.** alkaline phosphatase zymogram (cophenetic correlation coefficient r = 0.94832); **C.** esterase, acid and alkaline phosphatase, malate dehydrogenase, peroxidase and superoxide dismutase zymograms (cophenetic correlation coefficient r = 0.92866).

Colletotrichum species existing in coffee's microflora (namely *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*), is its pathogenicity towards developing coffee berries and seedling hypocotyls (Waller et al., 1993). Our data confirmed that all *C. kahawae* isolates are pathogenic to green coffee berries and supported the importance of these pathogenicity tests to distinguish *Colletotrichum* species. As previously reported (Várzea et al., 1993; Manga et al., 1997; Várzea et al., 1999; Omondi et al., 2000), the *C. kahawae* isolates showed variability in their aggressiveness independently of the geographic origin. The fast development of the symptoms together with the highest IDI values revealed that isolate Cam1 was the most aggressive. Similar results were obtained by Várzea et al. (1999). *C. kahawae* isolate Cam1 was the most aggressive isolate compared to other CBD isolates, due to a higher sporulation capacity and rapid conidial germination in host tissues. Additionally, our data also pointed that isolate Mal2 was the least aggressive.

The isoenzymatic analysis further supported the usefulness of the IEF technique for investigating enzyme polymorphisms and genetic diversity within *C. kahawae.* Polymorphisms were detected almost in all the enzymes analysed, especially EST, ALP and MDH. The EST system yielded the greatest number of bands and exhibited a high degree of polymorphism, being efficient to differentiate *C. kahawae* from *C. gloeosporioides*. In a previous work (Omondi et al., 1997), EST isoenzymes also provided the differentiation of *C. gloeosporioides*, *C. acutatum* and *C. kahawae* isolates from coffee. ACP, SOD, POD and MDH zymograms did not allow the separation of *C. kahawae* from *C. gloeosporioides*. These data corroborated to some extent the close relationship between *C. kahawae* and *C. gloeosporioides*, as previously shown in several molecular studies (Abang et al., 2002; Lubbe et al., 2004). The ALP enzyme system showed a high level of intraspecific variability related to *C. kahawae* aggressiveness (particularly Cam1 and Mal2) and was effective to differentiate the *C. gloeosporioides* isolate (Chi1) from *C. kahawae* isolates. Bridge et al. (2008) reported different AFLP patterns and VCG for isolates collected in Cameroon and Malawi compared to other *C. kahawae* isolates. Isoenzyme profile analysis has been useful to investigate genetic diversity within *Colletotrichum*. Lima Filho et al. (2003), using EST and ACP enzyme patterns obtained by PAGE, reported intraspecific variability among *C. gloeosporioides* isolates from several tropical fruits. Additionally, Rios et al. (2004) successfully used the EST, ACP, ALP and MDH isoenzymes, to confirm the existence of genetic variability in *C. lagenarium*.

Cluster I contained all *C. kahawae*, while cluster II had the isolate Chi1 from *C. gloeosporioides*. Although no correlation between geographical origin and the subgroups formed was found, this study was more successful in detecting intraspecific variation among *C. kahawae* isolates as well as interspecific variability than other molecular studies performed (Abang et al., 2002; Lubbe et al., 2004; Bridge et al., 2008; Manuel et al., 2010).

Despite the low number of isolates, the isoenzyme analysis using IEF was a useful tool to detect intraspecific variability among *C. kahawae* isolates and to separate *C. kahawae* from *C. gloeosporioides*. This study provides useful insights into the range of variability existing across these isolates. However, a more complete understanding of the genetic structure of *C. kahawe* populations would necessitate work on different geographical and spatial scales. From all the enzymatic systems studied, ALP was the most efficient to discriminate the two species and the only one apparently associated with aggressiveness. These studies are essential to improve coffee breeding, attaining higher levels of disease resistance towards a sustainable coffee production in African countries.

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